22-Oxa Calcitriol Is a Less Potent Regulator of Keratinocyte Proliferation and Differentiation Due to Decreased Cellular Uptake and Enhanced Catabolism

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22-oxa calcitriol (OCT) is a recently synthesized analog of calcitriol ($1,25(OH)_2D_3$) with potent biologic actions both *in vivo* and *in vitro*. Because it is considerably less hypercalcemic than $1,25(OH)_2D_3$ when given *in vivo*, OCT is of potential use for the treatment of diseases, such as psoriasis, that respond to the antiproliferative, prodifferentiating actions of $1,25(OH)_2D_3$. To determine the potential usefulness of OCT in hyperproliferative skin diseases, we compared the ability of OCT to that of $1,25(OH)_2D_3$ with respect to regulation of keratinocyte proliferation and differentiation *in vitro*. These studies were performed in serum-free media to eliminate differences in potency secondary to differences in binding to the serum vitamin D–binding protein. We observed that OCT was considerably less effective than $1,25(OH)_2D_3$ in inhibiting keratinocyte proliferation and stimulating differentiation. The decreased potency of OCT appeared to be due to decreased uptake and increased catabolism rather than decreased affinity for the vitamin D receptor. We conclude that under the conditions of our experiments OCT was less potent than $1,25(OH)_2D_3$ because it failed to achieve comparable concentrations within the cell. *Key words: transglutaminase/involucrin/cornified envelope/vitamin D receptor. J Invest Dermatol 105:693–698, 1995*

Although the classic role of $1,25(OH)_2D$ is to control calcium and phosphate homeostasis, acting principally on bone, gut, and kidney, it is now appreciated that $1,25(OH)_2D$ has receptors in and alters the function of a wide range of cells including the dominant cell of the epidermis, the keratinocyte (recently reviewed in [1,2]). Keratinocytes not only respond to exogenous $1,25(OH)_2D$ with changes in proliferation and differentiation [3–7], but produce $1,25(OH)_2D$ [8,9], suggesting that $1,25(OH)_2D$ may play an autocrine or paracrine role in the skin.

The ability of $1,25(OH)_2D_3$ to regulate epidermal proliferation and differentiation has been exploited clinically in the treatment of psoriasis [10,11]. However, because of the potential risk of hypercalcemia, hypercalciuria, and nephrocalcinosis when using $1,25(OH)_2D$ in normocalcemic individuals, analogs of $1,25(OH)_2D$ are being produced and studied in an effort to obtain compounds that retain the ability of $1,25(OH)_2D$ to inhibit proliferation and stimulate differentiation without stimulating intestinal calcium transport and bone resorption. Calcipotriene is one such analog that shows promise in the treatment of psoriasis [12,13]. We have studied another promising analog, 22-oxa calcitriol (OCT), for its potential efficacy in hyperproliferative skin diseases. OCT has been found to be at least as potent as $1,25(OH)_2D_3$ in stimulating differentiation and inhibiting proliferation of a variety of cells cultured in serum containing medium [14]. *In vivo*, OCT is effective in inhibiting parathyroid hormone secretion [15], decreasing tumor growth [16], and regulating the primary immune response [17].

Much of the apparent potency of OCT relative to $1,25(OH)_2D_3$ *in vitro* and its relative lack of hypercalcemic action *in vivo* appears to be due to comparable affinity of OCT and $1,25(OH)_2D_3$ for the intracellular vitamin D receptor (VDR), but much lower binding of OCT to the circulating vitamin D–binding protein (DBP) [18]. This results in a higher free concentration of OCT *in vitro* in serum-containing media and an accelerated clearance *in vivo* [19]. These properties would be expected to increase its potency relative to $1,25(OH)_2D_3$ *in vitro* when added to serum containing media [20] and decrease its potency and/or duration of action on intestinal calcium absorption and bone resorption *in vivo* [21].

In this study we compared the ability of OCT and $1,25(OH)_2D_3$ to regulate keratinocyte proliferation and differentiation using serum-free conditions to obviate differences in free concentrations. We anticipated comparable potency, and were surprised by the relative lack of effectiveness of OCT in this system. These results led us to consider the possibility that OCT and $1,25(OH)_2D_3$ underwent substantially different metabolic fates in keratinocytes. Such differences were found and appear to explain the much lower potency of OCT in keratinocyte cultures.

MATERIALS AND METHODS

Materials The OCT and ^{3}H-OCT were provided as gifts from Dr. Yasuho Nishii (Chugai Pharmaceuticals, Tokyo). The $1,25(OH)_2D_3$ was provided as a gift from Dr. Milan Uskokovic (Hoffmann-LaRoche, Nutley, NJ). ^{3}H-$1,25(OH)_2D_3$ was purchased from Amersham (Arlington Heights,

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Abbreviations: DBP, vitamin D–binding protein; OCT, 22-oxa calcitriol; VDR, vitamin D receptor.

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IL). The non-radioactive compounds were solubilized in ethanol and their concentrations determined from their ultraviolet spectra using an extinction coefficient at OD_{264} of 18,500. The radiolabeled compounds were purified by high-performance liquid chromatography (HPLC) prior to use using a Zorbax-Sil column (DuPont, Wilmington, DE) and a concave gradient of 97:3 to 90:10 hexane:isopropanol. The compounds were added to the cell cultures in ethanol to a final concentration of 0.5%.

Cell Culture Second-passage keratinocytes isolated from neonatal human foreskins were used in these studies. They were grown in serum-free keratinocyte growth medium obtained from Clonetics Corporation (San Diego, CA) as described previously [22] or in comparable medium from Gibco (Gaithersburg, MD). Details regarding individual experiments are found in the text and figure legends. In all cases the same keratinocytes were used for a given experiment comparing OCT to $1,25(OH)_2D_3$. Unless otherwise stated, the vitamin D compounds were added to the cells 5 d after plating, at which time the cells were approximately 70% confluent.

Cellular Proliferation ^{3}H-thymidine incorporation was determined as previously described [23] with the following modifications. The cells were grown in 96-well plates with 200 μl Gibco keratinocyte–serum-free medium containing 0.09 mM calcium for 48 h before the addition of the $1,25(OH)_2D_3$ or OCT. Incubation continued for up to 48 h. During the last 4 h, 1.5 μCi ^{3}H-thymidine was added to each well. The amount of radioactivity incorporated into trichloroacetic acid precipitable material was quantitated.

Cornified Envelope Synthesis This assay was performed as previously described [6]. The cells were grown for 5 d in Gibco medium containing 0.09 mM calcium. At this point the various concentrations of OCT or $1,25(OH)_2D_3$ were added. As an additional control some cells were switched to 1.2 mM calcium. Two microcuries per milliliter of ^{35}S-methionine was then added, and the cells continued their incubation for 48 h before harvest. Two hours before harvest the cells were exposed to 5×10^{-6} M ionomycin to maximize cornified envelope formation. The cells were then washed three times in phosphate-buffered saline, and the total radioactivity incorporated into trichloroacetic acid–precipitable material (total protein synthesis) as well as that incorporated into sodium dodecylsulfate (SDS)/dithiothreitol insoluble material (cornified envelopes), were determined.

Involucrin Protein Levels Cells were grown in Gibco medium containing 0.09 mM calcium until day 5 at which point various concentrations of OCT or $1,25(OH)_2D_3$ were added for an additional 48 h. At this point the cells were rinsed once with Ca^{++}, Mg^{++}-free phosphate buffered saline and then harvested in 2% SDS. Cell extracts were boiled and centrifuged at $12,000 \times g$ for 5 min to sediment-insoluble envelopes, and the supernatants were collected for protein measurement. Equivalent amounts of protein per sample were separated by SDS–polyacrylamide gel electrophoresis (PAGE) (8% polyacrylamide gel), blotted onto nitrocellulose paper, then immunostained using a polyclonal anti-involucrin (gift from Dr. Robert Rice). Positive reactions were visualized using the enhanced chemiluminescence kit from Amersham (Arlington Heights, IL) according to the manufacturer's instructions.

Transglutaminase Activity Transglutaminase activity was determined using the method of Schmidt *et al* [24]. Cells were grown for 5 d in Gibco medium containing 0.09 mM calcium, treated for 48 h with various concentrations of OCT or $1,25(OH)_2D_3$, then harvested by scraping into 50 mM Tris-HCl/5 mM ethylenediaminetetraacetic acid, pH 8.0, sonicated, and centrifuged at $12,000 \times g$ for 1 h. Cell pellets were resuspended into assay buffer (50 mM Tris-HCl, pH 8.0, 5 mM dithiothreitol, 10 mM $CaCl_2$, 5 mM ethylenediaminetetraacetic acid and 0.1% Triton X-100) containing 1 μCi ^{3}H-putrescine and 1 mg/ml methylated α-casein, and incubated at 37°C for 30 min. The reaction was stopped with the addition of 10% trichloroacetic acid, and the ^{3}H-putrescine incorporated into the precipitated casein was quantitated by scintillation spectroscopy.

Receptor Binding The cytosol receptor assay for $1,25(OH)_2D$ binding was performed as previously described [25]. The keratinocytes were extracted in buffer containing 50 mM Tris-HCl, 300 mM KCl, 1.5 mM ethylenediaminetetraacetic acid, 5 mM dithiothreitol, and 10 mM sodium molybdate, pH 7.4 (TKEDM), and the $100,000 \times g$ supernatant used as the source of the receptor. Three hundred eighty microliters cytosol (200 μg protein) were incubated for 8 h on ice with 10,000 cpm ^{3}H-$1,25(OH)_2D$ plus up to 3 nM OCT or $1,25(OH)_2D$ added in 20 μl ethanol. Unbound radioactivity was removed by charcoal extraction, and the radioactivity remaining in the supernatant was determined. Non-specific binding was subtracted from the results in performing the Scatchard analysis.

Total RNA Preparation and Northern Hybridization Total RNA was prepared according to the method of Chomczynski and Sacchi [26]. In brief, cells were dispersed in lysis solution (4 M guanidine isothiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) and then extracted with phenol-chloroform, followed by ethanol precipitation. For Northern analysis, RNA was electrophoresed through 1% agarose-formaldehyde gels, then transferred to Hybond-N^+ nylon membranes (Amersham, Arlington Heights, IL). The blots were hybridized with ^{32}P-labeled cDNA probes for transglutaminase (gift from Dr. Robert Rice), involucrin (gift from Dr. Howard Green), and an 18S RNA probe for normalization. Results were quantitated by densitometry of resulting autoradiograms.

Metabolism of OCT and $1,25(OH)_2D_3$ The keratinocytes were grown to confluence in keratinocyte growth medium containing 0.07 mM calcium. ^{3}H-OCT and ^{3}H-$1,25(OH)_2D_3$ (0.05 μCi/well) purified by high-performance liquid chromatography were added to the cell cultures for variable periods of time before the cells and media were separately collected, and the dish eluted with methanol to determine residual binding. Total radioactivity in the cells, media, and dish eluent were determined in 10% aliquots of the total; the remainder was then extracted and analyzed by high-performance liquid chromatography as previously described [9].

Statistical Analysis The data were analyzed by the appropriate programs in Statstix (Analytic Software, St. Paul, MN). In particular, the effects of the different vitamin D compounds were tested for significance by multivariate analysis of variance (MANOVA) that incorporated all data from the experiment in the analysis.

RESULTS

Comparison of OCT and $1,25(OH)_2D_3$ Inhibition of Keratinocyte Proliferation The ability of OCT and $1,25(OH)_2D_3$ to inhibit keratinocyte proliferation is shown in **Fig 1**. In this experiment, the cells were harvested 4, 8, and 24 h after the addition of 10^{-8} to 10^{-6} M OCT and $1,25(OH)_2D_3$. In other experiments, lower concentrations of these compounds showed no antiproliferative activity. As seen in this experiment, the inhibition of proliferation is drug, time, and dose dependent. $1,25(OH)_2D_3$ at the highest dose tested (10^{-6} M) inhibited proliferation 36% by 4 h and 87% by 24 h. OCT showed no significant inhibition at any dose at 4 h, but the highest dose (10^{-6} M) caused a 29% inhibition after 24 h. When analyzed by MANOVA the dose effect, drug effect, and time effect were all highly significant ($p < 0.0001$) as were the interactions between drug and dose ($p < 0.0001$) and time and dose ($p = 0.004$).

Comparison of OCT and $1,25(OH)_2D_3$ in Their Stimulation of Cornified Envelope Formation In this experiment **(Fig 2)** the vitamin D compounds were added 5 d after plating, and the cells were harvested 48 h later. The experiment was performed in media containing 0.09 mM calcium except for one set of cultures in which the calcium concentration was raised to 1.2 mM instead of adding one of the vitamin D compounds at the time the vitamin D compounds were added to the other cultures. These cells served as the positive controls. Increasing the $1,25(OH)_2D_3$ to 10^{-8} M resulted in an 88% increase in cornified envelope formation, comparable to the 71% increase caused by raising the calcium concentration. As analyzed by ANOVA, this stimulation was highly significant ($p = 0.0015$). In contrast, OCT did not significantly increase cornified envelope formation ($p = 0.10$). The difference between $1,25(OH)_2D_3$ and OCT in stimulating cornified envelope formation was highly significant ($p < 0.0001$) as assessed by multiple variable ANOVA.

Comparison of OCT and $1,25(OH)_2D_3$ in Their Stimulation of Transglutaminase Activity Transglutaminase activity is required for cornified envelope formation, crosslinking soluble substrates such as involucrin into the highly resistant cornified envelope. The experiment shown in **Fig 3** was performed using cells grown and treated as for the experiment depicted in **Fig 2**. As seen in **Fig 3**, $1,25(OH)_2D_3$ exerted a dose-dependent stimulation of transglutaminase activity ($p < 0.0001$), increasing the levels of activity 114% at 10^{-8} M. This was comparable to the 144% increase caused by the calcium switch. In contrast, OCT raised transglutaminase activity only 16% at 10^{-8} M ($p = 0.0415$). The differences

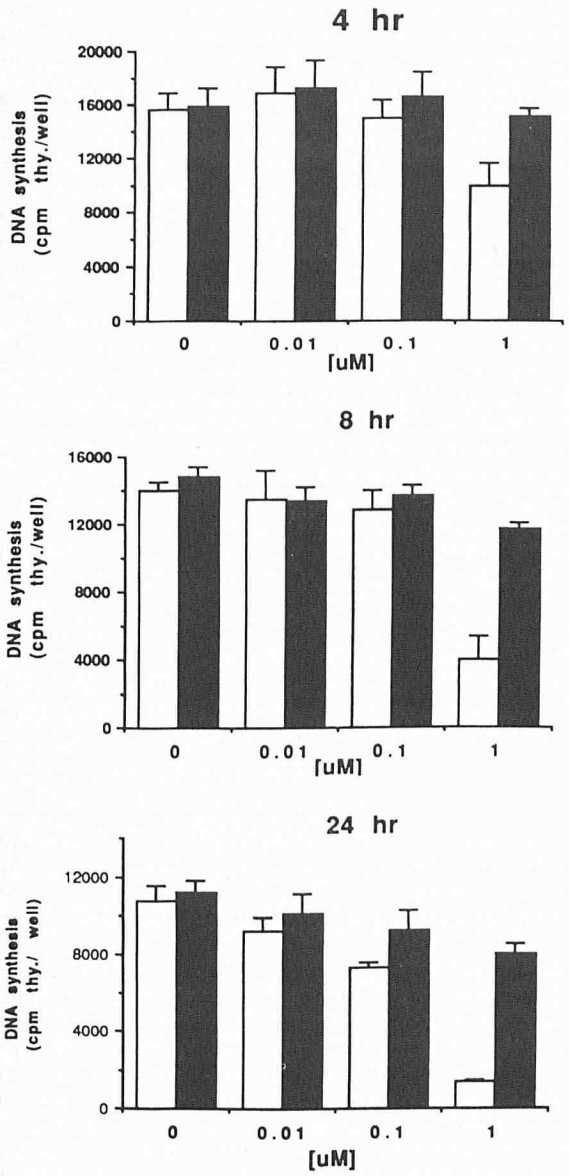


Figure 1. OCT is a less potent antiproliferative agent than $1,25(OH)_2D_3$. The cells were grown in 96-well plates in 0.09 mM calcium for 48 h before the addition of the indicated concentrations of $1,25(OH)_2D_3$ (*open bars*) or OCT (*closed bars*). Incubation continued for 4, 8, or 24 h before harvest. ^3H-thymidine was added during the last 4 h of the incubation. The cpm incorporated into the DNA is plotted. The data are expressed as mean ± SD of quadruplicate determinations. The effects of drug, dose, and time are all highly significant ($p < 0.0001$), with $1,25(OH)_2D_3$ being the more potent drug.

between $1,25(OH)_2D_3$ and OCT in their abilities to stimulate transglutaminase activity were highly significant ($p < 0.0001$).

Comparison of OCT and $1,25(OH)_2D_3$ in Their Ability to Raise Involucrin Levels Involucrin levels of cells treated for 48 h with $1,25(OH)_2D_3$ or OCT are shown in **Fig 4**. Involucrin is a 68-kD protein that runs aberrantly (i.e., as a higher–molecular-weight species) in SDS-PAGE. The 140-kD band is the major band immunoreacting with our anti-involucrin antibody, although a 90-kD band is also detected. The Western blot is shown in the upper panel; the densitometric tracing of the 140-kD band is shown in the lower panel. Involucrin levels increased twofold following incubation with 10^{-11} M $1,25(OH)_2D_3$ (from 0.7 to 1.6), and

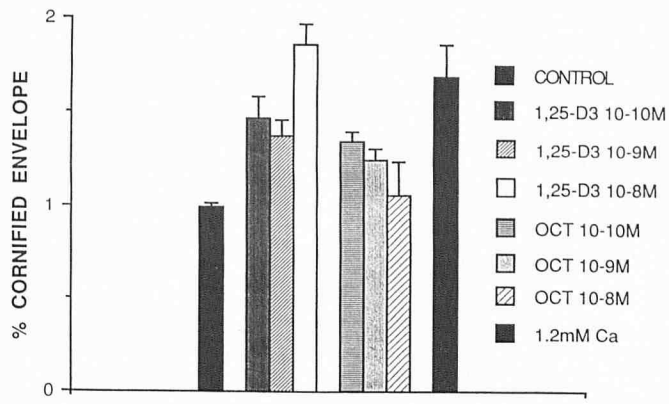


Figure 2. OCT is less potent than $1,25(OH)_2D_3$ in stimulating cornified envelope formation. The cells were grown in 0.09 mM calcium for 5 d before the addition of the indicated concentrations of OCT or $1,25(OH)_2D_3$ and ^{35}S-methionine for an additional 48 h. One set of cultures was switched to 1.2 mM calcium rather than receiving OCT or $1,25(OH)_2D_3$. The data are expressed as mean ± SEM of triplicate determinations. $1,25(OH)_2D_3$ but not OCT significantly stimulated cornified envelope formation ($p = 0.0015$ and $p = 0.0988$, respectively). The response to $1,25(OH)_2D_3$ was dose dependent ($p = 0.0004$) and significantly greater than that to OCT ($p = 0.0008$).

threefold (to 2.1) at the highest concentration of $1,25(OH)_2D_3$ evaluated (10^{-7} M). OCT also increased involucrin levels, but the stimulation at 10^{-7} M (1.2) was less than that observed at 10^{-11} M $1,25(OH)_2D_3$.

Comparison of OCT and $1,25(OH)_2D_3$ with Respect to Their Ability to Increase mRNA Levels for Transgluatminase and Involucrin The ability of $1,25(OH)_2D_3$ to increase involucrin and transglutaminase activity occurs by stimulation of gene transcription [27]. The cells were incubated with the indicated concentrations of OCT or $1,25(OH)_2D_3$ for 24 h before harvest and analysis of the mRNA levels. The results are shown in **Fig 5**. In this experiment the same blot was analyzed for involucrin mRNA, transglutaminase mRNA, and 18s RNA. The Northern analysis is shown in the upper panel. The lower panel shows the densitometric data normalized to 18s RNA. In this experiment, transglutaminase mRNA proved to be more responsive to both $1,25(OH)_2D_3$ and OCT than involucrin mRNA. However, $1,25(OH)_2D_3$ maximally stimulated the mRNA for involucrin

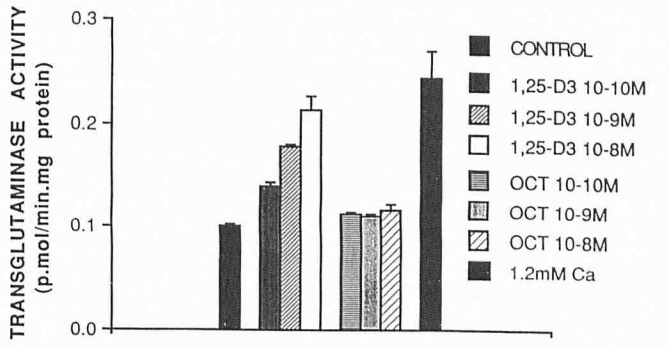


Figure 3. OCT is less potent than $1,25(OH)_2D_3$ in stimulating transglutaminase activity. The cells were grown as described in **Fig 2** and then treated for 48 h with the indicated concentrations of OCT, $1,25(OH)_2D_3$, or calcium. The results are expressed as mean ± SEM of triplicate determinations. The stimulation by $1,25(OH)_2D_3$ was significant ($p < 0.0001$), dose dependent ($p < 0.0001$), and greater than that by OCT ($p < 0.0001$); OCT had a slight but significant stimulatory effect ($p = 0.0415$).

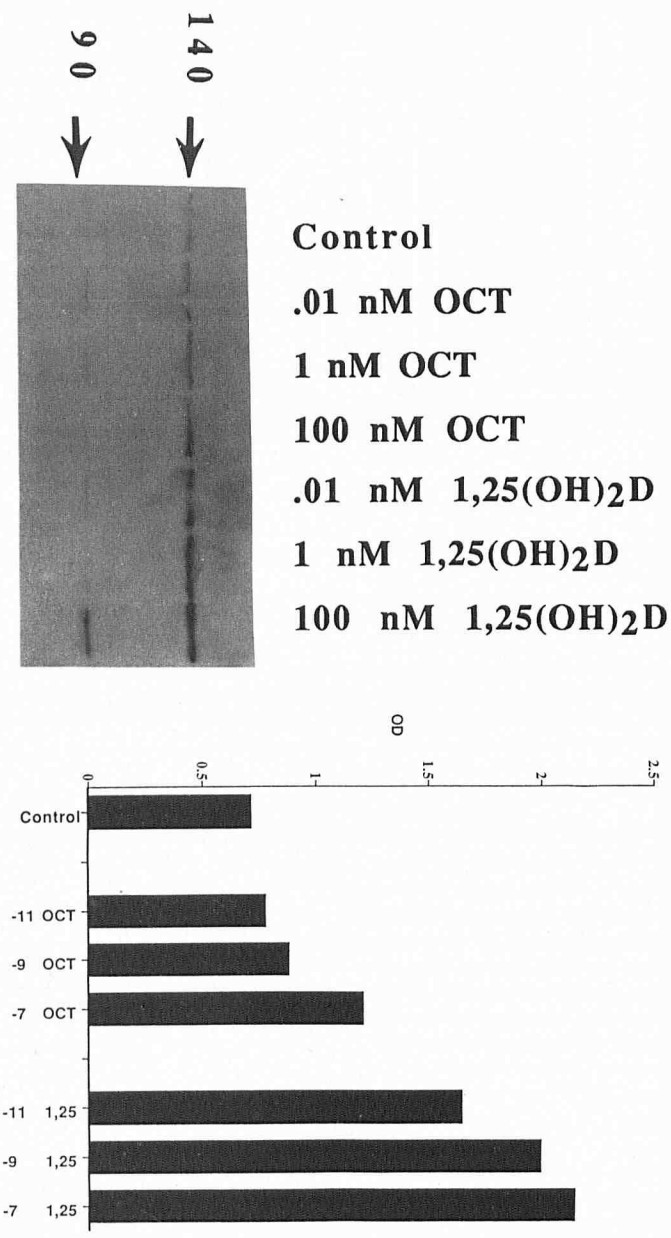


Figure 4. OCT is less potent than $1,25(OH)_2D_3$ in raising involucrin levels. The *top* shows the Western analysis; the *bottom* shows the results of densitometry. Only the results of the upper band are plotted in the bar graph. $1,25(OH)_2D_3$ stimulated involucrin levels more effectively than did OCT.

twofold (from 0.5 to 1.03) and the transglutaminase mRNA 5.3 fold (from 0.22 to 1.17) at 10^{-10} M. Higher concentrations were less effective. In contrast, OCT was maximally effective at 10^{-7} M with a 1.58-fold increase in involucrin mRNA and a 2.1-fold increase in transglutaminase mRNA. These are comparable to or less than the stimulation observed with 10^{-12}–10^{-11} M $1,25(OH)_2D_3$. The differences between OCT and $1,25(OH)_2D_3$ in stimulating the mRNA levels for involucrin and transglutaminase were significant ($p = 0.006$ and 0.003, respectively).

Affinity of OCT and $1,25(OH)_2D_3$ for the VDR To determine the reason for the markedly lower potency of OCT compared to $1,25(OH)_2D_3$ in the regulation of keratinocyte proliferation and differentiation we first compared the ability of OCT and $1,25(OH)_2D_3$ to bind to the intracellular VDR as measured by their displacement of ^3H-$1,25(OH)_2D_3$ from the VDR. These results are shown in **Fig 6**. When analyzed by the method of Scatchard, OCT had a Kd for the VDR of 215 pM, approximately twice that of $1,25(OH)_2D_3$ (113 pM). This difference would not be expected to account for the differences seen in the biologic responses.

Uptake and Metabolism of OCT and $1,25(OH)_2D_3$ We then assessed whether the metabolism of OCT in culture differed from that of $1,25(OH)_2D_3$. These results are shown in **Fig 7**. As seen in *A*, $1,25(OH)_2D_3$ disappeared from the medium at a faster rate than OCT. By 1 h, 65% of the $1,25(OH)_2D_3$ had been removed from the medium, and by 2 h only 19% of the original amount of $1,25(OH)_2D_3$ was left in the medium. In contrast, at 1 and 2 h 74% and 55% of the OCT remained in the medium. Within the first 2 h most of the $1,25(OH)_2D_3$ removed from the medium was found intact within the cell; of the total added, 69% and 58% of the $1,25(OH)_2D_3$ was found within the cells at 1 and 2 h, respectively **(Fig 7*B*)**. In contrast, the amount of intact OCT recovered within the cells failed to rise above 10% of the total added. After 2 h, the amount of recoverable OCT in medium plus cells fell below that of $1,25(OH)_2D_3$, indicating more rapid catabolism. After 4 h, most of the radioactivity following either $1,25(OH)_2D_3$ or OCT addition was found in the medium, but was no longer intact $1,25(OH)_2D_3$ or OCT. With time an increasing fraction of this radioactivity was found in the aqueous phase of the extraction. The levels of radioactivity bound to the dish did not exceed 15% following $1,25(OH)_2D_3$ addition and 10% following OCT addition throughout the 24 h experiment. Thus, it is apparent that OCT was less readily incorporated into keratinocytes and more readily catabolized under the conditions of these experiments.

DISCUSSION

OCT is a recently synthesized analog of $1,25(OH)_2D_3$ with limited hypercalcemic activity *in vivo* but potent effects on selected biologic actions of $1,25(OH)_2D_3$ including inhibition of parathyroid-hormone secretion [15], tumor-growth suppression [16], and immune regulation [17]. Many of the differences in activity between OCT and $1,25(OH)_2D_3$ *in vivo* and *in vitro* have been explained by their having comparable affinity for the intracellular VDR but different affinities for the DBP in blood [18–21,28]. *In vitro*, under culture conditions that include serum (and, thus, DBP), a higher free fraction of OCT would be available to the cell, and OCT would appear to be more potent than $1,25(OH)_2D_3$ [14,19,20]. Under serum-free conditions, OCT and $1,25(OH)_2D_3$ would be expected to have comparable levels of potency [29–31]. *In vivo*, OCT would be less bound to DBP and undergo a higher rate of clearance [19], resulting in a more transient biologic effect on intestinal calcium transport [21] or bone resorption. These expectations have been realized.

Because of the potential usefulness for a potent, non-hypercalcemic $1,25(OH)_2D_3$ analog such as OCT in the treatment of hyperproliferative skin diseases, we compared the abilities of OCT and $1,25(OH)_2D_3$ to regulate keratinocyte proliferation and differentiation. In this study we demonstrated the ability of $1,25(OH)_2D_3$ to suppress keratinocyte proliferation at doses less than that of OCT. This suppression required time, with little inhibition seen before 4 h, and was dose dependent with no suppression seen at concentrations of $1,25(OH)_2D_3$ less than 10^{-8} M. Such high concentrations exceed by several orders of magnitude the concentrations required to stimulate differentiation.

The process of differentiation examined, cornified envelope formation, is unique to the keratinocyte. This pathway involves the crosslinking of a number of unique proteins within the keratinocyte such as involucrin by a calcium-activated membrane-bound enzyme, transglutaminase, to form a highly resistant submembranous structure, the cornified envelope. The cornified envelope gives the terminally differentiated keratinocyte its name, corneocyte, and many of its important protective properties. $1,25(OH)_2D_3$ is established as an important regulator of keratinocyte differentiation [2–7]. We [32] have recently demonstrated that the ability of $1,25(OH)_2D_3$ to stimulate cornified envelope formation is due at

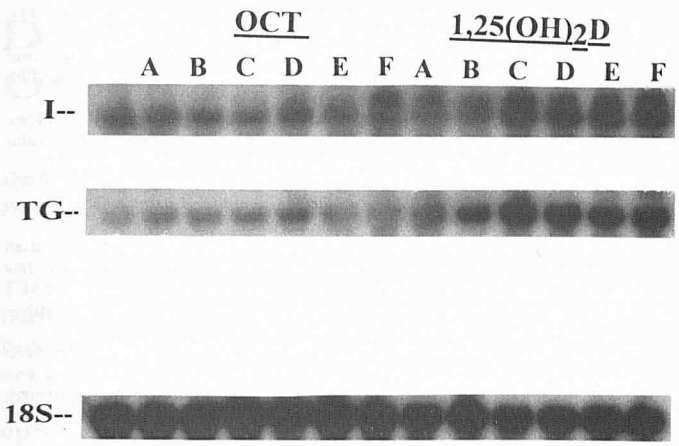
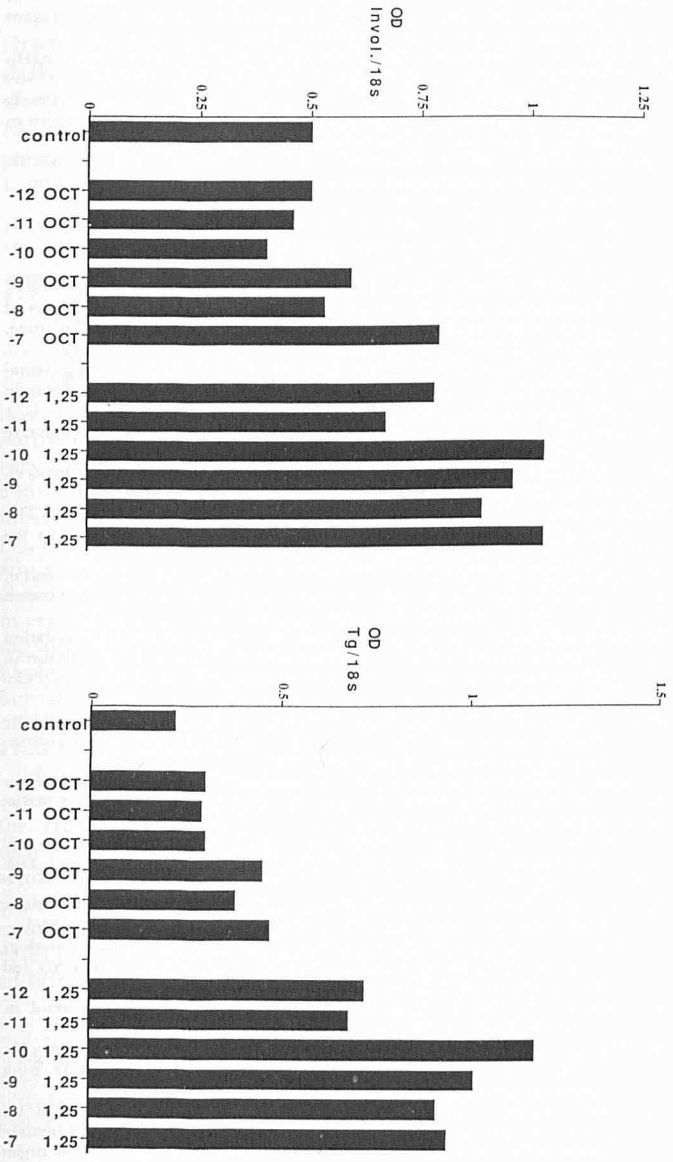


Figure 5. OCT is less potent than $1,25(OH)_2D_3$ in raising involucrin (I) and transglutaminase (TG) mRNA levels. Keratinocytes were grown in 0.07 mM calcium for 4 d, switched to 0.03 mM calcium, and the various concentrations of OCT or $1,25(OH)_2D_3$ added the following day. The cells were harvested 24 h later for RNA preparation. The same blot was probed for involucrin and transglutaminase mRNA, as well as 18s RNA for normalization. The unmarked lane on the left is the vehicle control. *Lanes A–F* represent increasing concentrations of OCT or $1,25(OH)_2D_3$ (10^{-12} to 10^{-8} M in 10^{-n} increments). The *top* contains the Northern data; the *middle and bottom* contain the data quantitated by densitometry and normalized to the 18s results. $1,25(OH)_2D_3$ stimulated the mRNA levels of involucrin and transglutaminase more effectively than did OCT ($p = 0.006$ and 0.003, respectively).



least in part to its stimulation of involucrin and transglutaminase gene transcription.

In the present study, $1,25(OH)_2D_3$ was found to stimulate cornified envelope formation, transglutaminase activity, and involucrin levels at concentrations of 10^{-10} M or less. The increase in mRNA levels at least for transglutaminase was even more sensitive with increases seen at concentrations of 10^{-12} M $1,25(OH)_2D_3$. OCT was considerably less potent than $1,25(OH)_2D_3$ in stimulating cornified envelope formation, involucrin levels, transglutaminase activity, and the mRNA levels for involucrin and transglutaminase; even at the highest concentrations of OCT studied (10^{-8}–10^{-7} M), stimulation of these proteins and activities was less than that seen following the lowest effective concentration of $1,25(OH)_2D_3$ examined (10^{-12}–10^{-10} M).

One potential explanation for the lack of potency of OCT in keratinocytes is low affinity for the keratinocyte VDR. However, this was not the case. As has been shown for other tissues [18,29,30,33], the VDR from keratinocytes binds OCT only slightly less well than it binds $1,25(OH)_2D_3$. Therefore, we evaluated the cellular uptake and catabolism of OCT in comparison to that of $1,25(OH)_2D_3$ to determine if reduced uptake or accelerated metabolism could contribute to the reduced potency of OCT. Our results demonstrate that OCT is not only less well taken up by the keratinocytes, but that it is more rapidly catabolized. The net result is that for comparable concentrations of OCT and $1,25(OH)_2D_3$ added to the cells, the intracellular concentrations of OCT never achieve a level greater than one seventh that of $1,25(OH)_2D_3$.

These observations in keratinocytes may not apply to all cell types because other cells do respond well to OCT, but cellular uptake and metabolism by other cell types have received little attention. In an experiment reported recently by Brown *et al* [21], vitamin D deficient rats were injected with radiolabeled OCT or $1,25(OH)_2D_3$. Although the serum level of OCT failed to reach the same level as that of $1,25(OH)_2D_3$, and disappeared more rapidly, the level of OCT in the chromatin from the intestinal mucosa was initially severalfold higher than that of $1,25(OH)_2D_3$. Calcium transport and mRNA levels for calbindin were both increased by OCT at least transiently in this experiment, comparable to that by $1,25(OH)_2D_3$. These data suggest that the intestinal cell may not limit entry of OCT, although the rapid decrease in OCT levels and

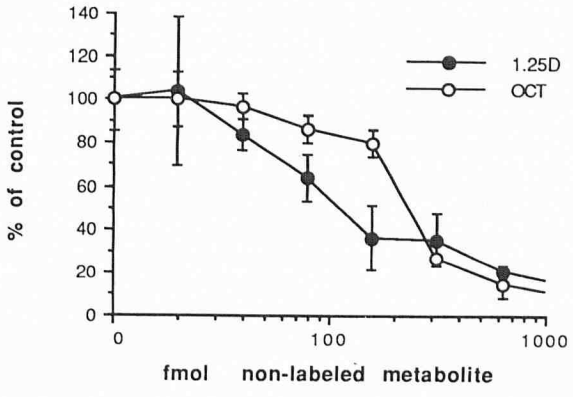


Figure 6. OCT has slightly lower affinity for the VDR than $1,25(OH)_2D_3$. The data show the comparison of the ability of increasing amounts of $1,25(OH)_2D_3$ and OCT to displace ^{3}H-$1,25(OH)_2D_3$ from the intracellular VDR. The data are expressed as mean ± SD of triplicate determinations.

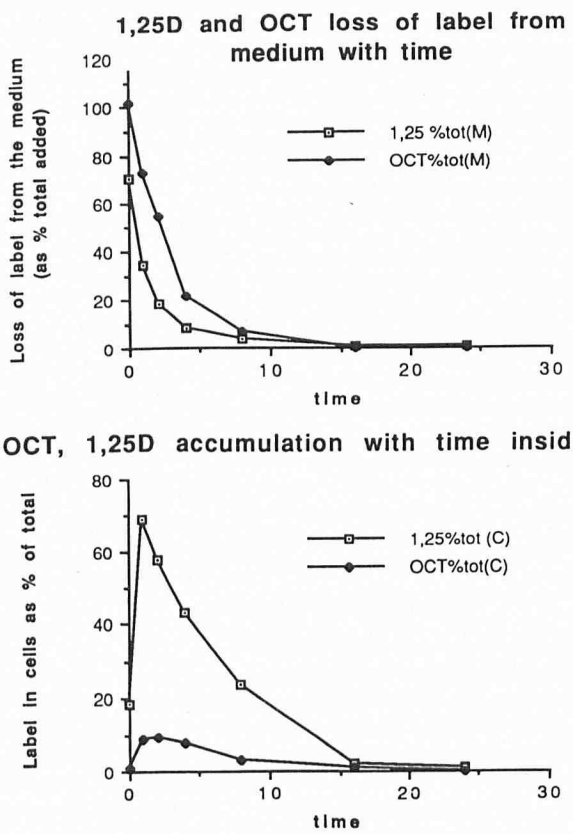


Figure 7. OCT is accumulated by keratinocytes less well than $1,25(OH)_2D_3$ and is catabolized more rapidly. The cellular uptake and metabolism of OCT and $1,25(OH)_2D_3$. Labeled OCT or $1,25(OH)_2D_3$ was added to the cells at time zero. The units of time are hours. The cells and media were extracted separately at the indicated times and analyzed for intact $1,25(OH)_2D_3$ or OCT by high-performance liquid chromotography. The *top* shows the levels of $1,25(OH)_2D_3$ and OCT in the medium; the *bottom* shows the levels in the cells. Each data point is the mean of duplicate dishes, the results of which were within 10% of the mean. $1,25(OH)_2D_3$ disappears faster from the media and achieves higher concentrations in the cell than OCT ($p < 0.0001$). Beyond 2 h less intact OCT than $1,25(OH)_2D_3$ remained in the incubate indicating that OCT was more rapidly metabolized than $1,25(OH)_2D_3$.

biologic effect in the intestine is consistent with increased metabolism. *In vivo* experiments are complicated by the role of DBP in regulating the free concentrations of the OCT and $1,25(OH)_2D_3$ available to the cell, however, so that differences in uptake of OCT and $1,25(OH)_2D_3$ cannot be excluded from these studies.

In conclusion, our results indicate that an interpretation of relative potencies of vitamin D analogs includes not only considerations of relative binding to DBP and VDR, but also an appreciation for different rates of cellular uptake and metabolism. In the keratinocyte, decreased uptake and increased catabolism limit the potency of OCT in regulating proliferation and differentiation.

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